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I. DIGESTION OF PRETREATED ASPEN SUBSTRATES: HYDROLYSIS RATES AND ADSORPTIVE LOSS OF CELLULASE ENZYMES

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ABSTRACT

Considerable controversy exists concerning the role lignin plays in the adsorption of cellulase enzymes on biomass. Recent studies using extracted, purified hardwood lignin have shown these materials have a propensity for cellulase adsorption; native lignin is, however, carbohydrate-linked and far less condensed. In this study we report the results of adsorption-kinetics analyses of cellulase-complex activities using five pretreated aspen substrates, including an exhaustively enzyme-hydrolyzed one. These data indicate that the polymer-binding cellulase activities are removed from solution at higher rates and extents in the presence of low lignin-content versus high lignin-content substrates. This order of adsorption was found to be essentially the inverse for beta-glucosidase adsorption.

INTRODUCTION

The reduction in cost of cellulase enzymes used for the saccharification of lignocellulosic feedstocks has been identified as a key goal in development of an economic, enzymatically based process for conversion of biomass to liquid fuels (Wright et al, 1986 and Wilke et al, 1976). Clearly, a significant cost reduction can be obtained by the recycle of saccharifying enzymes from the post-hydrolysis stream to the undigested feedstock (Isaacs, 1984). In practice, however, when enzyme-saturated hydrolysis residue is used as the vehicle for enzyme recycle, dramatic losses of potential activity against fresh feedstock are observed. These losses have been traditionally attributed both to non-specific binding of cellulase enzymes to lignin and to the highly specific binding of endoglucanases and cellobiohydrolases to residual cellulose (Sutcliffe et al, 1986; Deshpande et al, 1984; Klyosov et al, 1986; Ooshima et al, 1983; and Ryu et al, 1984). Furthermore, it has been reported that the adsorption of protein and enzyme are dependent on both the temperature (Ryu et al, 1983; Dove et al, 1981; Schulz et al, 1986; and Goel et al, 1983) and type of cellulosic material examined (Moloney et al, 1983; Lee et al, 1982; Mandels et al, 1981; Castanon et al, 1980; Lee et al, 1983); Lee et al. 1982b; Klysov et al, 1986; and Wilke et al, 1981). Recently published studies have dealt with the effects of various pretreatments of substrates (Lee et al, 1983; Lee et al, 1982b, Wilke et al, 1981, Sinitsyn et al, 1983, Puls et al, 1985; Mes-Hartree et al, 1986; Peitersen et al, 1977 and Rabinovich et al, 1982)[13, yet studies designed to target the role of native aspen lignin have not been reported.

In the work described in this paper, the adsorption of cellulase components on lignocellulosic feedstocks has been studied by means of two different but intersecting approaches. In the first approach, kinetic

experiments have determined the levels of Genencor 150L cellulase required to achieve maximum saccharification (initial rate and extent of hydrolysis) of the following pretreated aspen substrates: dilute sulfuric acid treated aspen meal (DAA), ethanol extracted DAA (EEDAA), sodium hydroxide (alkaline) extracted DAA (AEDAA), cellulase enzyme treated DAA (ENZDAA), and steam exploded aspen chips (SEA). In the second approach, the rate and extent of cellulase binding to the aspen substrates mentioned above has been evaluated in direct adsorption studies measuring the quantities of the various activities recoverable from supernatants in contact with these substrates. From these data, the role of in situ lignin in pretreated aspen feedstocks is evaluated.

EXPERIMENTAL

Preparation of aspen substrates. Wood meal (screened to 40-80 mesh) was produced by knife milling (with a Wiley mill model 2) chips obtained from a Foremost "Brushbandit" mobile chipper fed whole, debarked aspen poles. The aspen (Populus tremuloides) trees were harvested during the summer of 1985 on the eastern slope of the Colorado Rockies and inspected carefully to ensure freedom from fungal rot or insect infestations.

The DAA substrate sample was prepared in a 12-kilogram batch using a Pfaudler 50-gallon, glass-lined, stirred tank reactor. All subsequent chemical pretreatment experiments were carried out in a 1-L stirred reactor (Parr Instrument Co.) constructed from Carpenter 20Cb-3 stainless steel. The reactor was modified by addition of a pressurized acid injection device (Himmel, 1986) which allowed reagent addition after the reactor reached reaction temperature.

DAA was prepared by the hydrolysis of an 8% (w/w) slurry of aspen meal (18-80 mesh) with 0.45% (v/v) sulfuric acid at 140°C for 60 minutes. After cooking, the residue was washed with deionized water until the filtrate collected was neutral in pH and colorless. The EEDAA was prepared by a 12 hour Soxhlet extraction of 80-gram samples of DAA with spectral grade ethanol (Fisher). The AEDAA sample was prepared by a 2-hour, 140°C digestion of a 5% (w/w) slurry of DAA with 4% (w/v) NaOH. The residue was washed until the filtrate remained near neutral pH. The ENZDAA substrate was prepared by the exhaustive enzymatic digestion of DAA with Genencor 150L cellulase at 50°C in 50 mM citrate buffer, pH 4.8. To assure complete digestion, the enzyme was added in 3 additions of 25 FPU/gram substrate each, over a 7-day period. The residue was then filtered and washed with 50 mM citrate buffer to remove free cellulase enzyme. The residue was then extracted for 24 hours with 6M urea at 25°C to remove tightly adsorbed enzyme and then washed extensively with deionized water. The SEA sample was a generous gift from Dr. H. Brownell of Forintek Canada Corp. and was the result of the steam explosion of a 980-gram sample of aspen chips after an 80-second residence time at 240°C (470 psi gun pressure, no catalysts used). Like the other samples in this study, this sample was washed thoroughly before drying. The final substrate was alpha-cellulose obtained from Sigma Chemical Co. All samples were dried at 45°C for at least 24 hours before use. To assure uniformity and facilitate weighing, the "matted" samples were dispersed with a 30-second burst in a Bel-Art Micro-Mill (Fisher).

Analysis of pretreated substrates. Dry weights (i.e. air drying at 105°C to constant weight) and Klason-lignin were determined by standard methods, (Moore et al, 1967; and Official Test Methods, 1983), respectively. Ash and silica analyses were performed by gravimetric analysis according to AOAC methods (Horwitz, 1980). Anhydrosugars in the pretreated substrates were determined by a procedure slightly modified from that developed at the U.S. Forest Products Laboratory (Moore, 1967) as described previously (Grohmann et al, 1984).

Enzyme/enzyme assays. Assays for endo-beta-1,4 glucanase (carboxymethyl-cellulose degrading, CMCase) and saccharifying cellulase (filter paper units, FPU) activities followed the methods of Sternberg, Vijaykumar, and Reese (1977) and Mandels, Andreotti, and Roche (1976), as modified in the 1987 IUPAC report (Ghose et al, 1987). Under the recommended conditions of the CMCase and FPU assays, enzyme dilutions must be adjusted so that 0.5 mg and 2.0 mg glucose is released from 10 mg carboxymethylcellulose and 50 mg strips of Whatman No. 1 filter paper after 30 minute and one hour incubations at 50°C, respectively. Enzyme activities are then found from the required enzyme dilutions. General xylanase activity was estimated by the xylose released from oat spelts xylan (Sigma Chemical Co.) in 30 minutes at 50°C. Xylose released during this incubation was determined by the DNS method of Miller (1959). Beta-glucosidase was determined according to the method of Wood (1971) as aryl-beta-glucosidase by the hydrolysis of p-nitrophenyl-beta-glucopyranoside (Sigma Chemical Co.). The concentration of p-nitrophenol was determined from the extinction at 410 nm under alkaline conditions induced by the addition of 2 M Na_2CO_3. One unit of activity is defined as that amount of enzyme that catalyzes the cleavage of 1.0 micromol substrate per minute at 50°C. Beta-xylosidase activities were determined in a similar way using p-nitrophenyl-beta-xylopyranoside as substrate (John et al, 1979).

The cellulase enzyme preparation used in these studies was the Genencor 150L lot no. 3-86189 (code 6-5919) cellulase, found earlier (Himmel et al, 1986) by the above methods to have 109 FPU, 2842 CMCU, 288 beta-glucosidase units and 4.3 beta-xylosidase units of activity per mL.

In this study the enzyme assays described above were always performed in duplicate to ensure repeatability. Standard deviation was found routinely to be ±5% for filter paper and CMCase assays and ±3% for beta-glucosidase and beta-xylosidase assays.

Enzyme digestibility of pretreated substrates. All six pretreated aspen substrates were subjected to cellulase enzyme loadings of 5, 10, 20, 40, and 80 filter paper units (FPU) per gram cellulose in slurries of 1% (w/w) based on the cellulose content of the substrate. These digestion studies were carried out in 50 mL Nalgene polysulfone centrifuge tubes (known to exhibit low enzyme binding properties) equipped with o-ring sealed caps. These slurries were mixed by gentle agitation at 50°C. The glucose produced by 1, 6, 24, and 48 hours, and after 5 days was determined using the YSI Model 27 glucose analyzer.

Enzyme binding kinetics studies. The enzyme-substrate binding studies were carried out with all six pretreated substrates at an enzyme activity loading of 25 FPU/gram substrate and a solids concentration of 1.5% (w/w) in

50 mM acetate buffer, pH 5. For these studies enzyme loadings were based on units per gram total substrate (not on cellulose content). Slurries were loaded into 10 mL Nalgene vials and placed in a New Brunswick model L27 rotator for equilibration at 50°C and 4°C for 12 hours prior to enzyme addition. Genencor 150L cellulase was added to all vials after the 12-hour equilibration with acetate buffer; the vials were harvested by rapid filtration followed immediately by centrifugation at 1, 2, 5, 10, 20, 40, 60, and 180 minutes contact (with stirring by continued rotation) between enzyme and pretreated biomass. Filtration was performed with 5 mL disposable, polypropylene filters from Laboratory Supplies Co. and an Isolabs Inc. filtration chamber, followed by clarification of the supernatant by centrifugation for 3 minutes at 2,000 rpm in a Sorvall Instruments GLC-4 centrifuge. Aliquots of supernatant were then assayed for filter-paper-degrading, CMCase, xylanase, beta-glucosidase, and beta-xylosidase activities. Assays for "zero-time incubations" were obtained from samples treated identically except that the order of biomass removal (filtration and centrifugation) and enzyme addition was reversed; i.e., buffer and pretreated biomass were equilibrated at the appropriate temperature for twelve hours, biomass was removed by filtration and centrifugation, and then a mixture was made containing both enzyme stock and equilibrated, filtered and centrifuged buffer, such that the concentration in the activity assay mixture of both enzyme and any substances extracted from the biomass during equilibration would be equivalent to those present in the test samples after thorough mixing of the liquid components, but before any adsorption of enzyme had taken place.

RESULTS AND DISCUSSION

The samples described in this study represent lignocellulosic material (aspen wood) that has been subjected to five different pretreatment procedures intended to enhance the subsequent enzyme digestibility. Four of the pretreatments began with dilute sulfuric acid hydrolysis of the aspen wood meal; the fifth pretreatment involved only the steam explosion of the aspen chips. The enzyme digestibilities of the various pretreated substrates are shown in Figures 1 and 2, along with that of alpha-cellulose, which was used as a control substrate. The curves in Figure 1 show that alkali-extracted dilute-acid aspen (AEDAA) and alpha-cellulose substrates were readily hydrolysed, closely followed by steam-exploded aspen (SEA). The ethanol extracted (EEDAA) substrate (data not shown here) exhibited essentially the same digestion time-course as the DAA substrate. Both EEDAA and DAA were much poorer substrates for enzyme digestion than the other substrates studied. At 80 FPU/gram substrate, AEDAA and alpha-cellulose reached 100% digestion after 48 hr and SEA at 120 hr, whereas EEDAA and DAA had not quite reached 70% hydrolysis even at 120 hr.

The ordering of the digestibilities of all five substrates is even more evident in Figure 2, in which the initial digestion rates (found graphically from data given in Figure 1 using tangents drawn to the initial two data points in each curve) are plotted as a function of enzyme loading. The initial rates for DAA and EEDAA have essentially reached their maximum values at 40 FPU/gram substrate, whereas at an enzyme loading as high as 80 FPU/gram substrate, the initial rates for SEA, alpha-cellulose and AEDAA are all still increasing with enzyme loading. From this observation, we may infer the presence of far more sites for the initial attack in the latter three

substrates. However, we have noted earlier that air drying, even at 45°C for 24 hours, causes dilute acid treated aspen to become less digestible by cellulase enzymes than if it is not allowed to dry after pretreatment. To a certain extent, then, the differences in digestibility may reflect differing sensitivities of the substrates to low temperature drying.

Most models for the digestion of cellulose assume an initial, specific binding of certain enzyme components to the surface of insoluble substrate. In addition, it has been suggested that enzyme components may be bound non-specifically either to the cellulose itself or to the lignin component of lignocellulosic material, both of which result in removal of enzyme activity from the solution and a decrease in the activity available to carry out the hydrolysis of the cellulose. The studies described thus far (Figures 1 and 2) have dealt with digestibilities of various substrates; the remaining studies will focus on initial adsorption on these substrates of the different enzyme activities, as measured by the loss of the activities from the solution.

As noted by Lee (1982), Orichowsky (1982) and others, and as will be shown later in this paper, binding of the enzyme components to the substrates involves in each case an initial rapid adsorption process, which is essentially complete by 20 min after addition of enzyme to substrate. By withdrawing aliquots from the supernatant during the first 20 minutes, as well as at 40 and 60 minutes, we were able to study this initial binding step without complications due to changes in substrate structure that would be expected with significant hydrolysis, and were also able to avoid interference with subsequent enzyme assays by glucose and other products or inhibitors (as observed by Sutcliffe and Saddler (1986) produced during incubation of enzyme with pretreated substrates.

Figures 3 through 7 illustrate the time-course of removal of enzyme activities (loading: 25 FPU/gram substrate = 0.375 FPU/mL of solution) from solutions in contact with the various substrates. The enzyme loading chosen is one that is meaningful with respect to actual use of cellulase in digestions, since this loading is shown to give reasonable digestion rates for all of the pretreated aspen substrates studied (Figures 1 and 2). Furthermore, from an experimental point of view, this loading represents a practical middle ground between enzyme concentrations too low to give usable signals, and enzyme concentrations so high as to reduce the usable sensitivity of the subtractive method (i.e., "activity bound" equals activity added minus activity found in solution after contact) used in estimating the amount of enzyme bound to the various substrates. The enzyme activities reported in Figures 3-5 are given in terms of the amount of reducing sugar (glucose or xylose) released in one hour from the polymeric substrate. Over the narrow ranges of glucose release shown in Figures 3 and 4, the proportionality between glucose release and cellulase concentration (dilution) was found experimentally to be roughly linear (data not shown). Thus, in studies such as this one, in which the objective is to determine relative orderings, or trends, among the various samples, glucose release is a useful measure of cellulase activity. Furthermore, it should be kept in mind that in studies such as these the reduction in solution activity is not due to a simple dilution in equal proportions of all of the components of the cellulase mixture. The reduction in solution activity is due, rather, to the apparently selective adsorption of various components on the lignocellulosic biomass

which will change the ratios of the various component activities left in solution. For this latter reason, the expression of solution activity in terms of the primary data, i.e., reducing sugar release, carries fewer unwarranted implications than does expression of the residual activity as filter-paper units, since these units are essentially an expression of the simple dilution that must be made from a given cellulase stock in order to obtain a specified glucose release.

Figure 3 demonstrates the effects of different treatments on the time-course of adsorption of saccharifying enzymes (as measured by filter paper assays) on the pretreated substrates at different temperatures. The upper panel shows the adsorption found at 4°C. The ENZDAA (circles) presented a minimum of binding sites to the cellulase enzyme system as little loss in measured filter paper activity was found even after one hour in contact with this substrate. SEA (asterisks) showed considerable rapid adsorption in the first ten minutes (up to 20% loss in glucose released in subsequent filter-paper assays on the supernatant) as compared to the zero time value. Additional adsorption, though at a much slower rate, continued even after the one hour contact period. AEDAA (triangles) showed an adsorption curve similar to that of SEA but with a lesser degree of adsorption. Although all six substrates demonstrated biphasic binding curves (indicating multiple types of binding), steady-state conditions were achieved in most cases after ten minutes of contact at 4°C. Raising the temperature to 50°C (lower panel figure 3) did not substantially affect the degree of binding or the time at which maximum adsorption was found (within ten minutes) with the exception of commercial alpha-cellulose. The binding of the cellulase enzymes to alpha-cellulose nearly coincides with that of AEDAA. Both at 4°C and 50°C the ENZDAA showed very little adsorption, while the SEA showed the highest adsorption.

Figure 4 shows the adsorption of the endoglucanase components (as measured by CMCase assays) on the six substrates as a function of time spent in contact with these substrates. The top panel shows the adsorption at 4°C. SEA and AEDAA showed greater endoglucanase adsorption rates even after 60 minutes, while the other four substrates showed less than 4% loss in activity as compared to the zero time value. Again, the adsorption of the four other substrates is essentially complete within ten minutes. A larger percentage of activity loss was found at 50°C (lower panel figure 4) for all six substrates as compared to that found at 4°C. The solution in contact with SEA showed approximately 17% loss in endoglucanase activity at 60 min. The other five substrates showed less than 10% loss of the endoglucanase activity contained in the Genencor 150L cellulase.

Deshpande and Eriksson (1984) have found that the cellulolytic enzymes have a high affinity for native lignin in wheat straw (i.e., ~50% adsorption), yet our results for aspen indicate less than 20% and 10% adsorption for filter paper and CMCase activities, respectively. These discrepancies may be attributed, in part, to the differences between aspen and straw lignins.

Figure 5 shows the adsorption of xylan-degrading components of the Genencor 150L cellulase as a result of contact for various time intervals with the six substrates at 50°C. Most of the adsorption occurred well before 20 minutes. SEA adsorbed the greatest proportion of the xylanase (approximately 12%) after 60 minutes, while the other five substrates clustered near 6%

adsorption at the same time.

Certain broad similarities are evident in the results for the polymer-degrading enzyme activities. The binding behavior of xylanase mirrors that of CMCase, in that the steam-exploded substrate (SEA) binds both activities much more strongly than do the other four substrates, which show essentially the same ordering of binding behavior for both activities. With the exception of the results for ENZDAA (which show removal of almost no filter-paper activity from the solution), the results for filter-paper binding are similar, though in this case greater variability is seen between the extents of binding by different substrates.

The binding kinetics for beta-glucosidase are shown in Figure 6 at 4°C (upper panel) and 50°C (lower panel). Those substrates (ENZDAA, DAA, EEDAA, and SEA) that have significant lignin contents bind beta-glucosidase to a greater extent than those substrates (alpha-cellulose and AEDAA) that have very small lignin contents. Furthermore, binding by the substrates with significant lignin contents shows a much greater temperature dependence than does binding by those with very low lignin contents. The percentage of beta-glucosidase removed by the high-lignin substrates increases by an average of 16 percentage points upon the increase of temperature from 4°C to 50°C. The low-lignin substrates, on the other hand, show binding-percentage changes of 7 and -1 upon increase of temperature from 4°C to 50°C. This difference in temperature-dependence is consistent with a significant contribution from hydrophobic bonding between beta-glucosidase and lignin (perhaps utilizing the enzyme-active-site aglycone binding pocket).

The binding of beta-xylosidase activity at 50°C shown in Figure 7, illustrates striking differences from that of beta-glucosidase binding (Figure 6, lower panel). The order of beta-xylosidase binding showed no relationship to anhydroxylose contents or to lignin contents (see Table 1). The ENZDAA and DAA show about 20% binding at 50°C for both the beta-glucosidase and beta-xylosidase. The SEA, alpha-cellulose, and AEDAA binding curves for beta-xylosidase provide a dramatic contrast with up to 70% binding found as compared to approximately 20% binding for the beta-glucosidases to these substrates. Binding is also significantly slower for beta-xylosidase taking nearly 60 minutes as opposed to 20 minutes for beta-glucosidase.

Figure 8 shows correlations between substrate-lignin content (Table I) and the relative effectiveness of removal from solution of one polymer-degrading activity, filter-paper activity, (from Figure 3) and beta-glucosidase activity (from Figure 6). The apparent binding of filter-paper activity was shown to decrease with increasing substrate-lignin content, whereas adsorption of beta-glucosidase activity increases with increasing substrate-lignin content. The latter observation is in agreement with the recent findings of Sutcliffe and Saddler (1986).

As noted earlier, the steam-exploded substrate (SEA) differs from the other four aspen substrates studied in that the first step in pretreatment was not hydrolysis by dilute sulfuric acid at intermediate temperatures. The SEA substrate can further be distinguished by the observation that it showed by far the highest binding of polymer-degrading cellulase activities, yet it was not the most rapidly hydrolyzed to glucose. Also, the SEA was not found to agree with the trends shown in Figure 8, which relate lignin content to beta-

glucosidase and cellulase adsorption, in that the level of enzyme adsorption to SEA was higher than predicted from the five other substrates studied. This result indicates that the steam explosion process may generate a more "reactive" lignin, which possesses the high enzyme binding properties observed by previous workers (Sutcliffe and Saddler, 1986) for in vitro (or extracted) lignin.

Although these data indicate a potential justification for delignification of pretreated aspen to minimize beta-glucosidase loss, native lignin appears to have little effect on the substrate-specific adsorption of cellulose depolymerizing enzymes.

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Table 1

CHEMICAL ANALYSIS OF PRETREATED ASPEN SUBSTRATES

Substrates	% Klason Lignin	% Anhydroglucose	% Anhydroxylose
ENZDAA	66	34	-0-
DAA	29	65	2
EEDAA	25	67	4
SEA	24	66	5
AEDAA	2	98	-0-
alpha-cellulose	-0-	97	3
aspen wood*	18	50	18

*For comparison

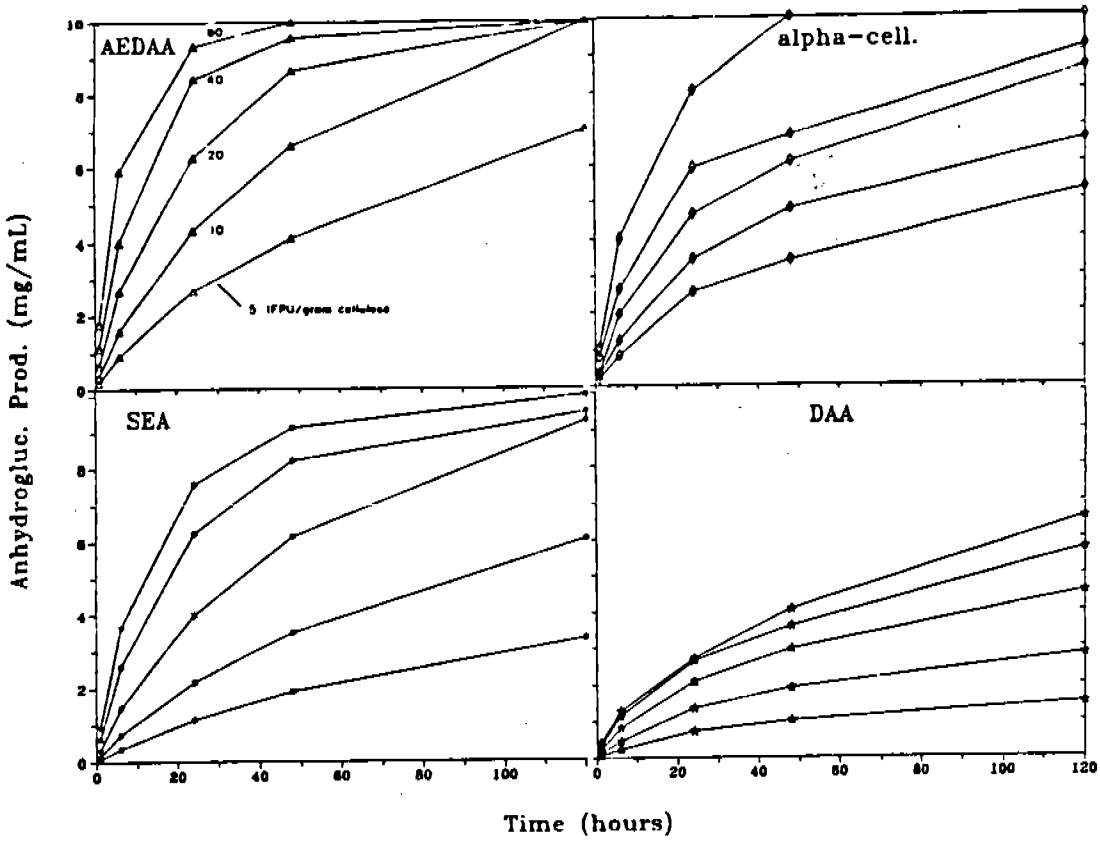

Figure 1. Digestibilities of three pretreated aspen substrates and alpha-cellulose with Genencor 150L cellulase at 50°C. Slurries were designed to contain 1% (w/w) cellulose from chemical analysis data. Cellulase loadings were 5, 10, 20, 40, and 80 FPU per gram cellulose content for each substrate and are indicated in increasing order as five curves of anhydroglucose release. A yield of 10 mg/mL was taken as 100%. Substrate code used for Figures 1-7 is: asterisk, SEA; circle, ENZDAA; square, EEDAA; triangle, AEDAA; diamond, alpha-cellulose; and star, DAA.

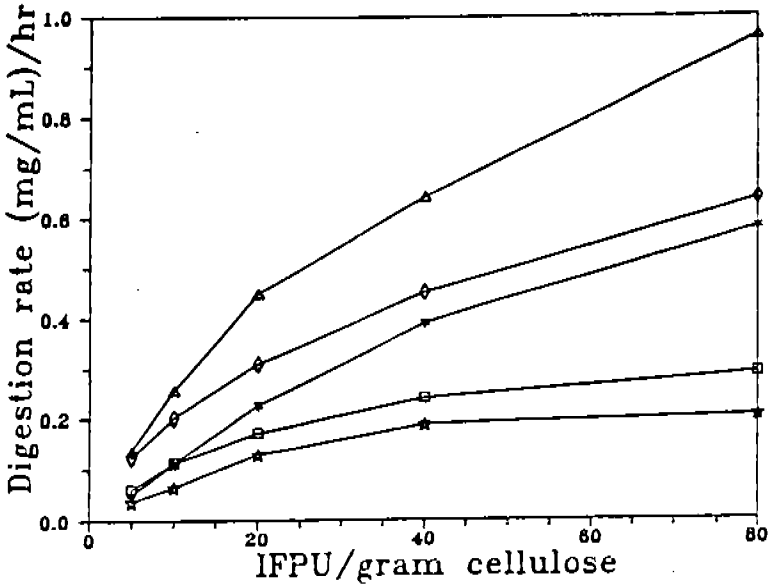

Figure 2. Initial rates of digestion for five substrates treated with Genencor 150L cellulase at 50°C. Values were estimated from curves in Figure 1 by graphical approximation using tangent lines drawn to the initial two data points. Symbols as in Figure 1.

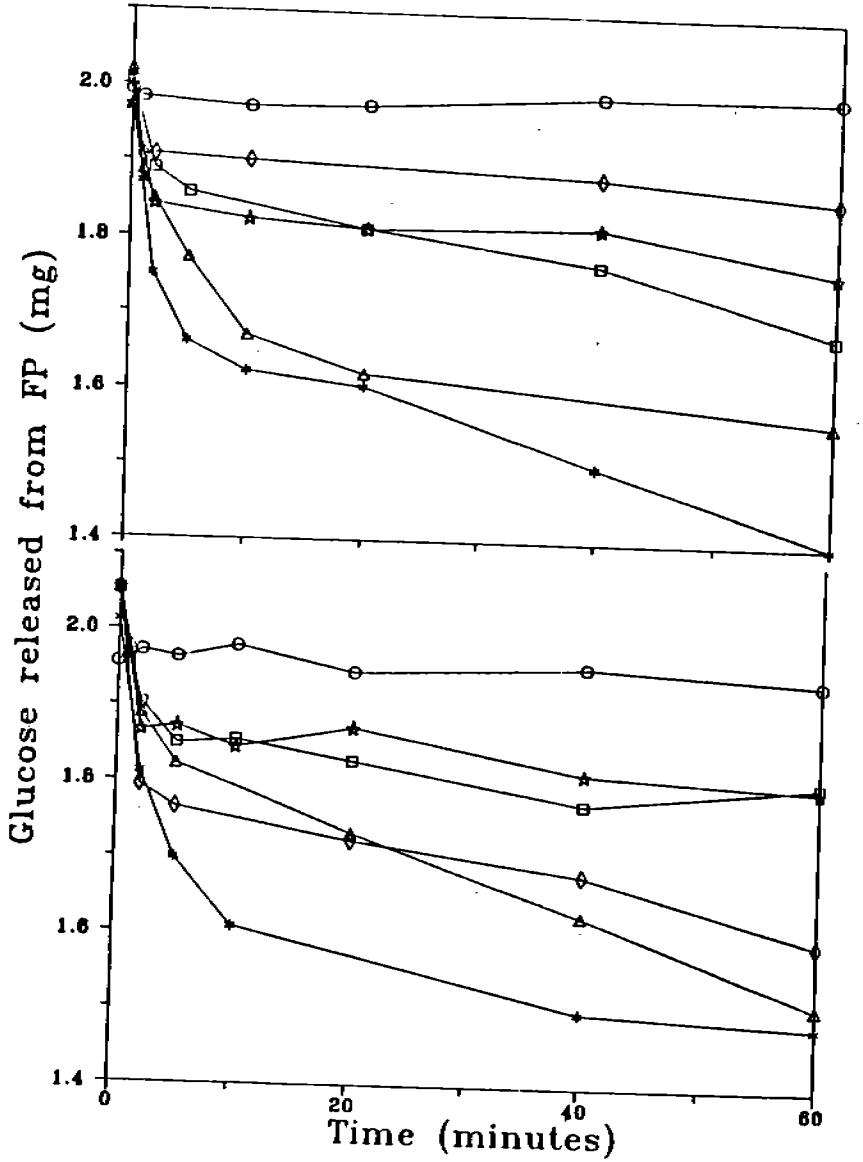

Figure 3. Binding kinetics for filter-paper-degrading enzymes with the five pretreated aspen substrates and alpha-cellulose at 1.5% (w/w) solids (free solution activities shown). Incubation temperature was 50°C (lower panel) and 4°C (upper panel). The initial enzyme loading was 25 FPU/gram substrate. Symbols as in Figure 1.

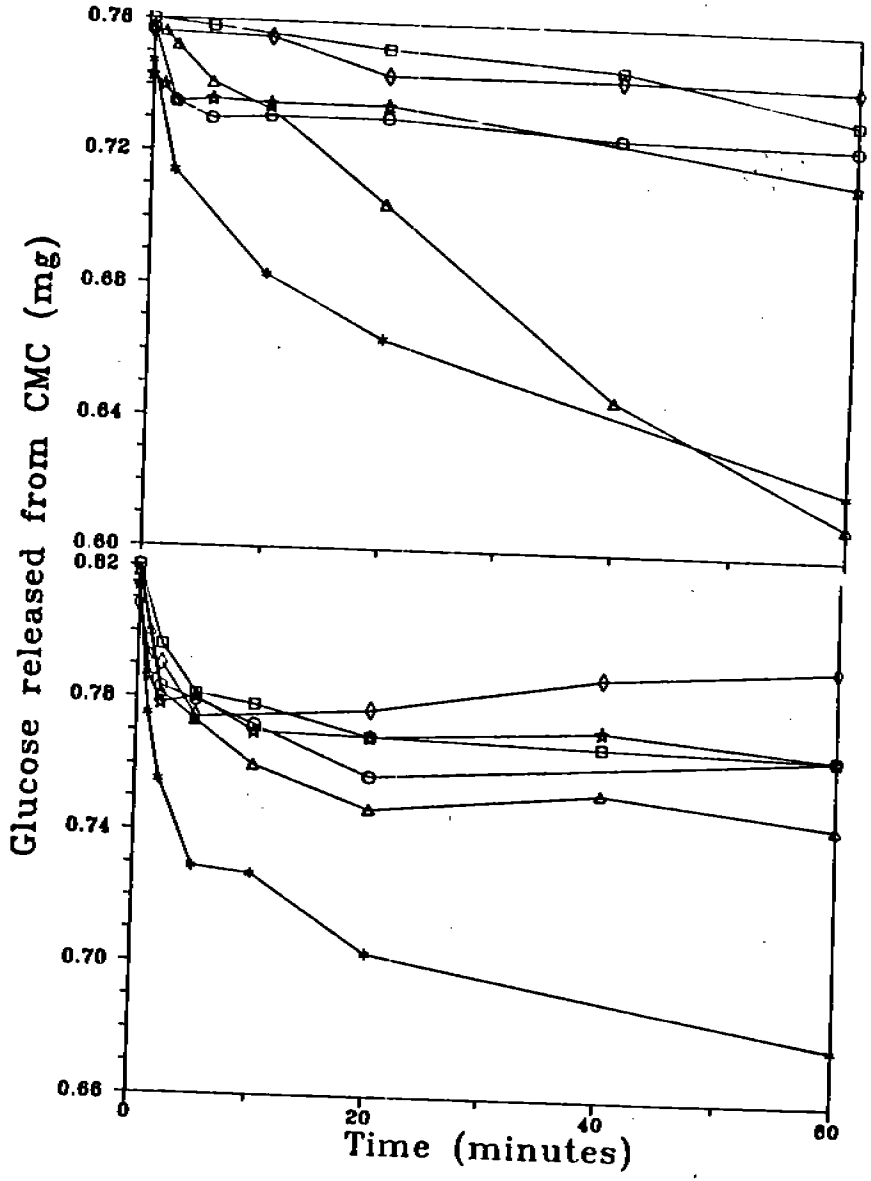

Figure 4. CMCase binding kinetics with five pretreated aspen substrates and alpha-cellulose at 50°C (lower) and 4°C (upper). Conditions and symbols as in Figure 3.

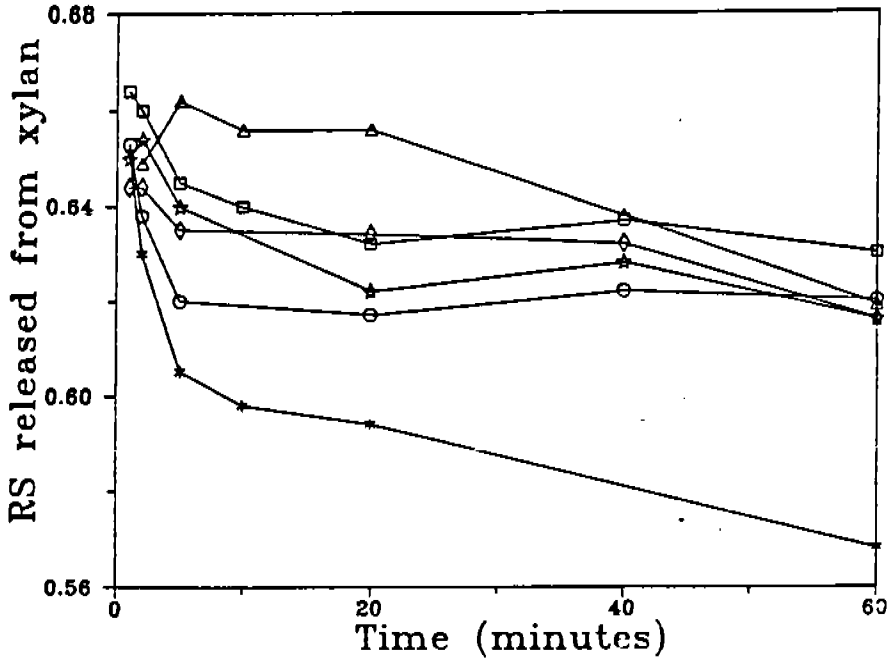

Figure 5. Xylanase binding kinetics with six substrates at 50°C. Enzyme activity was determined by following the release of xylose (mg per assay) from xylan using reducing sugar (RS) detection. Conditions and symbols as in Figure 3.

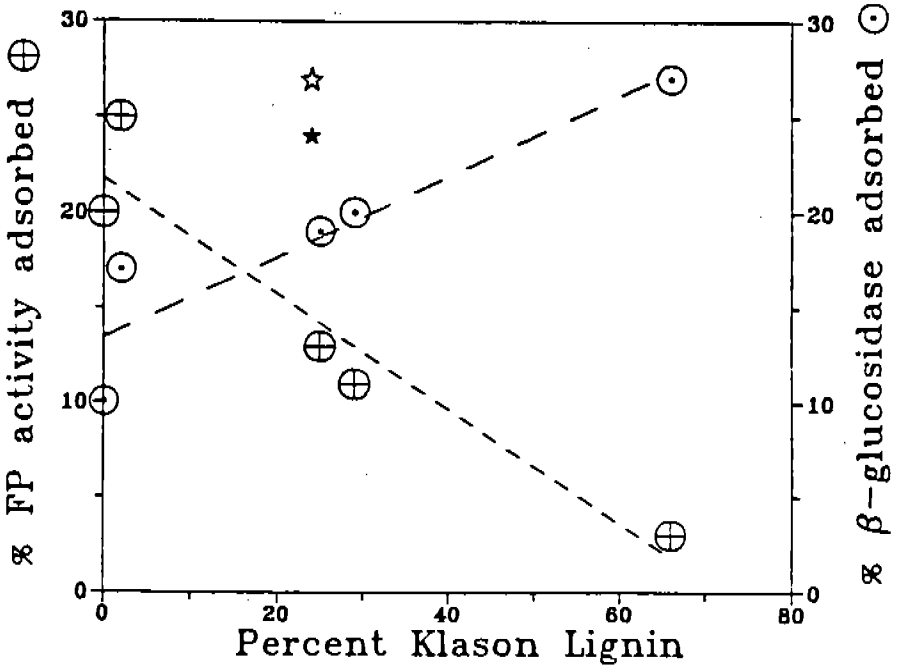

Figure 8. Correlations between Klason-lignin contents of substrates and the relative degree of activity loss after one hour exposure to substrate. The filter paper binding data is shown by circled crosses and the beta-glucosidase binding data by circled dots. The open and closed stars show binding data for the SEA filter paper and beta-glucosidase, respectively. The dashed lines represent a linear least squares fit to the data (SEA data excluded).

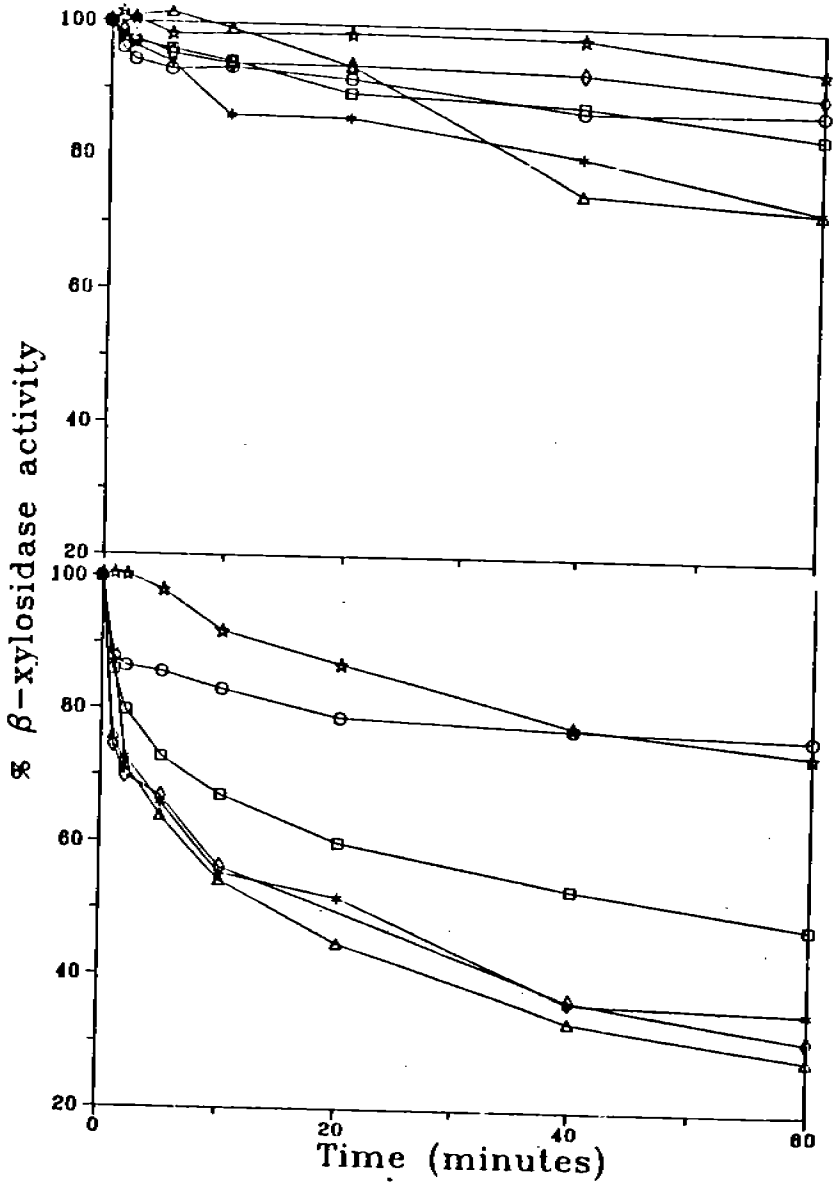

Figure 6. Beta-glucosidase binding kinetics with six substrates at 50°C (lower) and 4°C (upper). Conditions and symbols as in Figure 3.

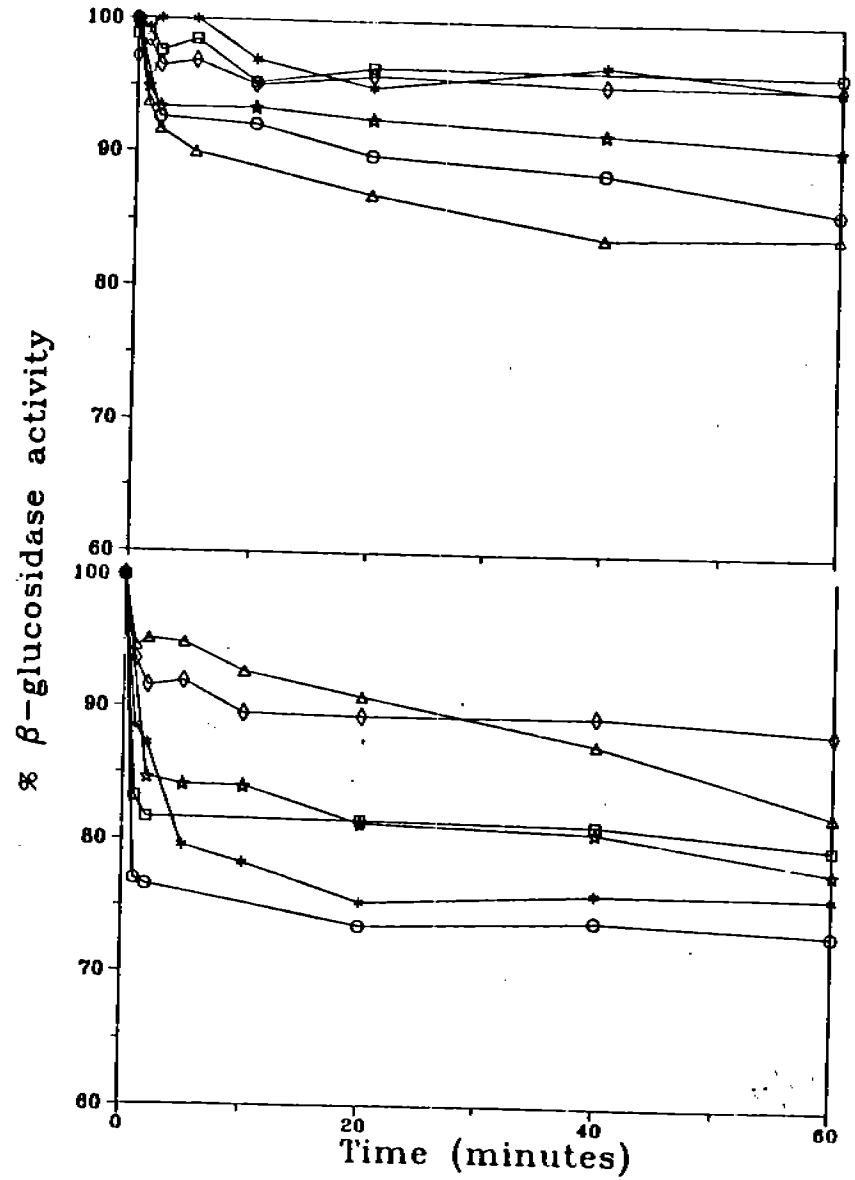

Figure 7. Beta-xylosidase binding kinetics with six substrates at 50°C (lower) and 4°C (upper). Conditions and symbols as in Figure 3.